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## Constitutive Signaling of the Human Cytomegalovirus-encoded Chemokine Receptor US28\*

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Previously it was shown that the HHV-8-encoded chemokine receptor ORF74 shows considerable agonist-independent, constitutive activity giving rise to oncogenic transformation (Arvanitakis, L., Geras-Raaka, E., Varma, A., Gershengorn, M. C., and Cesarman, E. (1997) *Nature* 385, 347–350). In this study we report that a second viral-encoded chemokine receptor, the human cytomegalovirus-encoded US28, also efficiently signals in an agonist-independent manner. Transient expression of US28 in COS-7 cells leads to the constitutive activation of phospholipase C and NF- $\kappa$ B signaling via  $G_{q/11}$  protein-dependent pathways. Whereas phospholipase C activation is mediated via  $G_{q/11}$  subunits, the activation of NF- $\kappa$ B strongly depends on  $\beta\gamma$  subunits with a preference for the  $\beta_2\gamma_1$  dimer. The CC chemokines RANTES (regulated on activation, normal T cell expressed and secreted) and MCP-1 (monocyte chemotactic protein-1) act as neutral antagonists at US28, whereas the CX<sub>3</sub>C chemokine fractalkine acts as a partial inverse agonist with IC<sub>50</sub> values of 1–5 nM. Our data suggest that a high level of constitutive activity might be a more general characteristic of viral G protein-coupled receptors and that human cytomegalovirus might exploit this G protein-coupled receptor property to modulate the homeostasis of infected cells via the early gene product US28.

Viruses have developed a variety of strategies to evade the immune system, among which is the piracy of cellular genes that are central to the host defense system (1–3). The identification of a variety of viral genes that encode potential G protein-coupled receptors (GPCRs)<sup>1</sup> or GPCR ligands is in this respect of major interest as the GPCR superfamily is essential for proper cellular communication (4). In the genome of various  $\beta$ - and  $\gamma$ -herpesviruses, like human cytomegalovirus (HCMV or HHV-5) (5–7), human herpesviruses HHV-6 (8–10), HHV-7

(11), and HHV-8 (Kaposi's sarcoma-associated *Herpesvirus*) (12–15) viral genes with homology to mammalian chemokines and/or chemokine receptors have been identified. These observations suggest that these viruses exploit chemokine signaling pathways to interfere with the host immune system (1–3).

Currently, the best characterized viral GPCR is ORF74, a CXCR2 homologue encoded by HHV-8 that binds a variety of CXC and CC chemokines (16–18). ORF74 signals in a chemokine-independent, constitutively active manner (16–18) and induces oncogenic transformation when transfected in NIH-3T3 cells (19). Although constitutive GPCR signaling is now a well accepted paradigm, the actual physiological relevance is still not entirely understood (20, 21). HHV-8 is considered to be the etiologic agent of Kaposi's sarcoma, a highly vascularized tumor (22). As transgenic expression of ORF74 also results in angioproliferative lesions, resembling various symptoms of Kaposi's sarcoma (23), the constitutive activity of ORF74 is one of the intriguing examples of a potential pathophysiological role of constitutive GPCR signaling.

In this study we report that a second viral-encoded chemokine receptor, the HCMV-encoded GPCR US28, also efficiently signals in an agonist-independent manner. The  $\beta$ -herpesvirus HCMV has been recognized as a risk factor for vascular diseases, like arterial restenosis and atherosclerosis, and causes life-threatening systemic infections in immunocompromised patients (24, 25). Sequence analysis of the HCMV genome has identified four genes encoding GPCRs, US27, US28, UL33, and UL78 (5), of which US28 is expressed early after viral infection (26). US28 shows the highest homology (33%) to the CC chemokine receptor CCR1 and binds CC chemokines, like RANTES and MCP-1 (6, 27), and the CX<sub>3</sub>C chemokine fractalkine (27). US28 shows considerable HIV-I coreceptor activity (28, 29) and is known to enhance *in vitro* cell-cell fusion mediated by various viral proteins, including HIV-I envelope proteins (30). Moreover, US28 has been shown to induce vascular smooth muscle cell migration (31), which could provide the molecular basis for the implication of HCMV in atherosclerosis.

In this study, we show that upon transient expression in COS-7 cells US28 constitutively couples to phospholipase C and NF- $\kappa$ B via related, though distinct,  $G_{q/11}$ -protein-mediated mechanisms. Our data suggest that a high level of constitutive activity might be a more general characteristic of viral GPCRs and that HCMV might exploit this general GPCR property to modulate the homeostasis of infected cells via the early gene product US28.

### EXPERIMENTAL PROCEDURES

**Materials**—ATP disodium salt, bovine serum albumin, chloroquine diphosphate, DEAE-dextran (chloride form), and pertussis toxin (PTX) were obtained from Sigma. D-Luciferin was purchased from Duchefa Biochemie B. V. (Haarlem, The Netherlands). Cell culture media, penicillin, and streptomycin were obtained from Life Technologies, Inc.,

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<sup>1</sup> The abbreviations used are: GPCR(s), G protein-coupled receptors; HCMV, human cytomegalovirus; HHV, human herpesvirus; HIV-I, human immunodeficiency virus, type I; NF- $\kappa$ B, nuclear factor- $\kappa$ B; PTX, pertussis toxin; CMV, cytomegalovirus; InsP, inositol phosphate.

and fetal calf serum was purchased from Integro B. V. (Dieren, The Netherlands). *myo*-[2-<sup>3</sup>H]inositol (17 Ci/mmol) was obtained from PerkinElmer Life Sciences. The human chemokines RANTES (regulated on activation, normal T cell expressed and secreted), MCP-1 (monocyte chemoattractant protein-1), GRO- $\alpha$ , IP-10, and the CX<sub>3</sub>C chemokine domain of human fractalkine (residues 1–76) were obtained from Peprotech (Rocky Hill, NJ).

**DNA Constructs**—pNF- $\kappa$ B-Luc was obtained from Stratagene (La Jolla, CA). The cDNAs encoding for US28 (encoded by VHL/E HCMV strain) and US28-N (encoded by AD169 HCMV strain) (GenBank<sup>TM</sup> accession numbers L20501 and X17403, bases 219,000–220,263) inserted into pcDNA3 were a gift from Dr. R. Doms. The cDNA of the HHV-8-encoded ORF74 in pTJE8 was a gift from Dr. T. Schwartz. The cDNA of ORF74 was inserted in pcDNA3 after polymerase chain reaction amplification. Gifts of pcDNA3-based expression vectors containing the cDNAs of CCR1 (from Dr. C. Tensen), muscarinic m2 receptor (from Dr. R. Maggio), G $\alpha_q$  (from Dr. B. Conklin), G $\alpha_{11}$  and G $\alpha_{11}$ Q209L (from Dr. H. Umemori), G $\alpha_s$  and G $\alpha_{12}$  (from Dr. G. Milligan), G $\alpha_{16}$  (from Dr. S. Rees), G $\alpha_{12}$  (from Dr. N. Dhanasekaran), G $\alpha_i$  (from Dr. B. De-fize), G $\beta_1$ , G $\beta_5$ , G $\gamma_1$ , and G $\gamma_2$  (from Dr. M. Lohse), and GRK2 and GRK2K<sup>220R</sup> (from Dr. S. Cotecchia) are gratefully acknowledged.

**Cell Culture and Transfection**—COS-7 cells were grown at 5% CO<sub>2</sub> at 37 °C in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, 50 IU/ml penicillin, and 50  $\mu$ g/ml streptomycin. Transfection of the COS-7 cells was performed by DEAE-dextran. The total amount of DNA in transfected cells was maintained constant by addition of the empty vector.

**[<sup>3</sup>H]Inositol Phosphate Production**—Cells were seeded in 24-well plates, and 24 h after transfection they were labeled overnight in inositol-free medium (modified Eagle's medium with Earle's salts) supplemented with 2 mM L-glutamine, L-cysteine, L-leucine, L-methionine, L-arginine, glucose, 0.2% bovine serum albumin, and 2  $\mu$ Ci/ml *myo*-[2-<sup>3</sup>H]inositol in the presence or absence of PTX (100 ng/ml). Subsequently, the labeling medium was aspirated, cells were washed for 10 min with Dulbecco's modified Eagle's medium containing 25 mM HEPES (pH 7.4), 20 mM LiCl, and incubated for 2 h in the same medium in the absence or presence of the tested chemokines. The incubation was stopped by aspiration of the medium and addition of cold 10 mM formic acid. After 90 min of incubation on ice, inositol phosphates were isolated by anion exchange chromatography (Dowex AG1-X8 columns, Bio-Rad) and counted by liquid scintillation.

**Reporter-gene Assay**—Cells transiently cotransfected with pNF $\kappa$ B-Luc and either pcDNA3 (mock) or pcDNA3-US28 were seeded in 96-well black plates (Costar) in serum-free culture medium in the presence or absence of PTX (100 ng/ml) and the tested chemokines. After 48 h, cells were assayed for luminescence by aspiration of the medium and addition of 25  $\mu$ l of luciferase assay reagent (0.83 mM ATP, 0.83 mM D-luciferin, 18.7 mM MgCl<sub>2</sub>, 0.78  $\mu$ M Na<sub>2</sub>H<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 38.9 mM Tris (pH 7.8), 0.39% (v/v) glycerol, 0.03% (v/v) Triton X-100, and 2.6  $\mu$ M dithiothreitol). Luminescence was measured for 3 s in a Wallac Victor<sup>2</sup>.

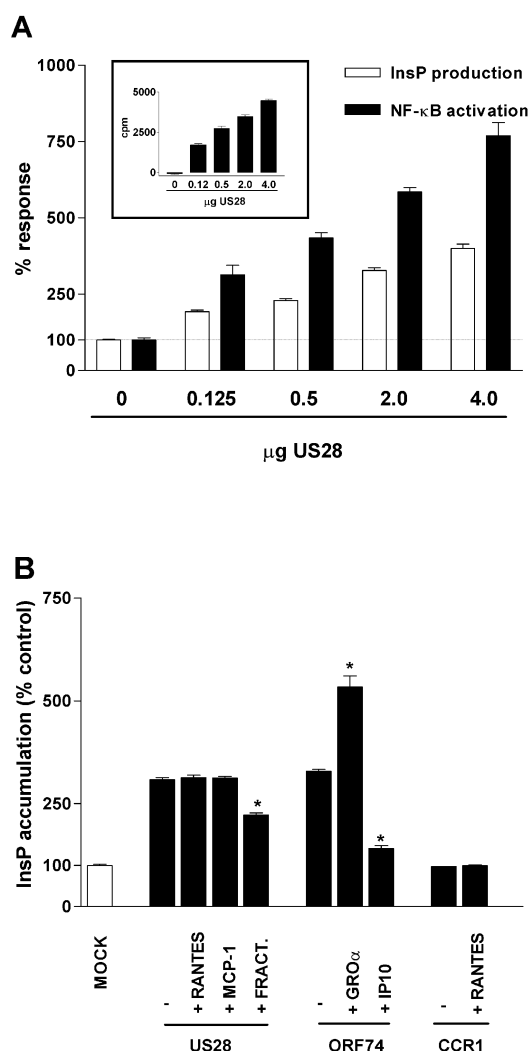
**Binding Experiments**—Cells were seeded in 24-well plates; 48 h after transfection binding was performed on whole cells for 3 h at 4 °C using 0.1 nM [<sup>125</sup>I]-RANTES in binding buffer (50 mM Hepes, pH 7.4, 1 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, and 0.5% bovine serum albumin). After incubation, cells were washed four times at 4 °C with binding buffer supplemented with 0.5 M NaCl. Nonspecific binding was determined in the presence of 0.1  $\mu$ M cold competitor (RANTES or fractalkine).

**Western Blot Analysis**—Cells were lysed 48 h after transfection in RIPA buffer (phosphate-buffered saline containing 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonylfluoride, and 2  $\mu$ g/ml of aprotinin and leupeptin), sonicated, separated by SDS polyacrylamide gel electrophoresis, and blotted to polyvinylidene difluoride membrane. An antibody recognizing the common motif of G $\beta$  (Sigma) was used in combination with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody. Protein bands were detected with ECL chemiluminescence and quantified using an ImageStation (PerkinElmer Life Sciences).

**Statistical Analysis**—All data shown are expressed as mean  $\pm$  S.E. Statistical analysis was carried out by Student's *t* test. *p* values < 0.05 were considered to indicate a significant difference.

## RESULTS AND DISCUSSION

Expression of the viral chemokine receptor US28 (encoded by the HCMV VHL/E strain) in COS-7 cells resulted in an expression-dependent increase in both [<sup>3</sup>H]inositol phosphate production and NF- $\kappa$ B activation (Fig. 1A). Expression of the

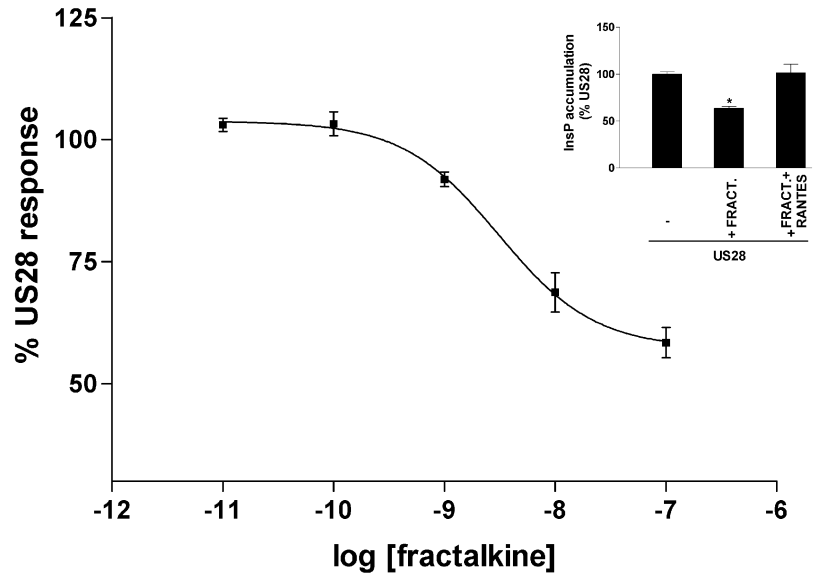


**FIG. 1. US28-mediated induction of NF- $\kappa$ B activity and inositol phosphates accumulation.** A, COS-7 cells ( $1 \times 10^6$  cells) were transiently transfected with increasing amounts of cDNA encoding US28. 48 h after transfection InsP accumulation and NF- $\kappa$ B-driven luciferase expression were measured. *Inset*, increase of specific [<sup>125</sup>I]RANTES binding to COS-7 cells after transfection of increasing amounts of US28-cDNA. B, modulation of inositol phosphates accumulation by various chemokines. COS-7 cells were transiently transfected with cDNAs encoding US28, ORF74, or the CCR1 receptor (2  $\mu$ g/ $10^6$  cells). Cells were incubated with the indicated chemokines (100 nM), and InsP production was measured. Data are presented as percentage of control (mock cells). Representative experiments performed in triplicate are shown; each experiment was repeated at least twice. The asterisks indicate a statistically significant difference (*p* < 0.05) versus receptor only. *FRACT.*, fractalkine.

related US28-N, encoded by the HCMV AD169 strain, gave similar findings (data not shown). As reported in Ref. 16, the expression of the HHV-8-encoded oncogenic GPCR ORF74 also led to a pronounced constitutive activation of phospholipase C (Fig. 1B). In contrast, expression of the human CCR1 receptor, which is most homologous to US28 (6), did not result in constitutive or RANTES-mediated phospholipase C activation (Fig. 1B). These findings are in accordance with previous observations that activation of CCR1 does not result in [<sup>3</sup>H]inositol phosphates accumulation in COS-7 cells (32).

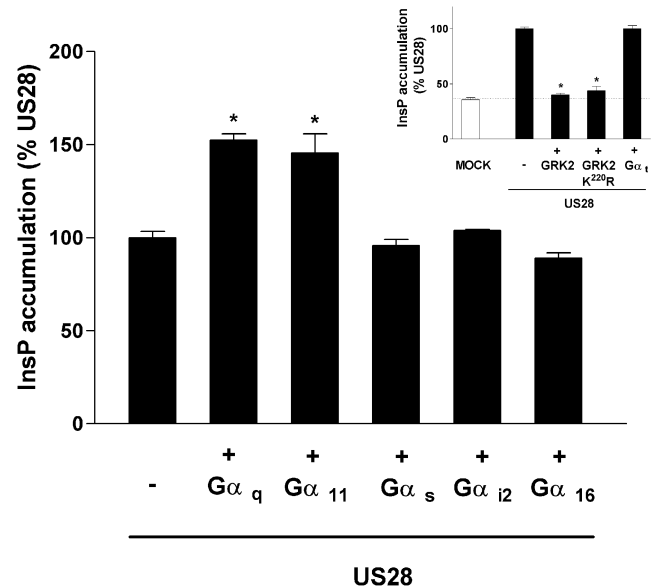
The constitutive activity of ORF74 was negatively modulated by IP-10 and positively by GRO- $\alpha$  (Fig. 1B), thus acting as inverse agonist and agonist, respectively (17, 18). The US28-induced signaling was not affected by the CC chemokines RANTES or MCP-1 up to 100 nM but was inhibited by the CX<sub>3</sub>C chemokine fractalkine (Fig. 1B). It has been reported that

**FIG. 2. Inhibition of US28-mediated inositol phosphates accumulation by fractalkine.** COS-7 cells were transiently transfected with cDNA encoding US28 (2  $\mu\text{g}/10^6$  cells). Cells were incubated with various concentrations of fractalkine, and InsP release was measured. *Inset*, US28-transfected cells were incubated with fractalkine (10 nM) in the presence or absence of RANTES (100 nM, added 10 min prior to fractalkine), and InsP accumulation was measured. Data are presented as percentage of US28-mediated response and defined as absolute increase of US28-mediated InsP accumulation above values obtained for mock-transfected cells. A representative experiment of three experiments, each performed in triplicate, is shown. The asterisk indicates a statistically significant difference ( $p < 0.05$ ) versus receptor only. *FRACT.*, fractalkine.



fractalkine binds to US28 (27), but so far no functional activity of fractalkine at US28 has been described. Fractalkine inhibited the constitutive US28 signaling to phospholipase C by  $37 \pm 4\%$  with an  $\text{IC}_{50}$  value of  $1.6 \pm 0.2$  nM ( $n = 3$ ; Fig. 2). As the observed inhibition of the US28 signaling is not complete, fractalkine apparently behaves as a partial inverse agonist (20, 21). The CC chemokines RANTES (100 nM) (Fig. 2, *inset*) and MCP-1 (data not shown) antagonized the reduction of basal US28 signaling by 10 nM fractalkine, thereby acting as neutral antagonists. RANTES and MCP-1 have previously been shown to act as agonists at US28 for  $\text{G}_i$ -dependent signal transduction (6, 33). These data can be explained by differences in the levels of constitutive US28 signaling in the different cell systems, probably as a result of differences in expression of US28 and/or signaling moieties (20, 21). Because  $\text{G}_i$  proteins are known signaling partners for chemokine receptors (34), including US28 (6, 33), the activation of phospholipase C by US28 could be due to the release of  $\text{G}\beta\gamma$  subunits, which can activate phospholipase C isoenzymes (35). Yet,  $\text{G}\alpha_i$  subunits are not implicated in the US28-mediated constitutive activation of phospholipase C. PTX treatment did not abolish the US28-mediated production of [ $^3\text{H}$ ]inositol phosphates ( $103 \pm 5\%$ ;  $n = 3$ ), whereas for the muscarinic m2 receptor PTX treatment inhibited the carbachol-induced increase in [ $^3\text{H}$ ]inositol phosphate accumulation for  $45 \pm 1.8\%$ . Coexpression of  $\text{G}\alpha_{i2}$  with US28 did not increase the US28 response (Fig. 3), probably because of the absence of the  $\beta\gamma$ -sensitive phospholipase  $\text{C}\beta_2$  in COS-7 cells (32). Instead, coexpression of US28 with either  $\text{G}\alpha_{11}$  or  $\text{G}\alpha_q$  enhanced the US28-mediated production of [ $^3\text{H}$ ]inositol phosphates (Fig. 3), whereas coexpression of  $\text{G}\alpha_{16}$  or  $\text{G}\alpha_s$  did not affect US28 responsiveness. Previously, the receptor kinases GRK2 and -3 have been reported to scavenge both  $\text{G}\alpha_{q/11}$  subunits (36), as well as  $\beta\gamma$  subunits (37). Coexpression of US28 with GRK2 or the kinase-deficient GRK2K<sup>220R</sup> (38) mutant resulted in an efficient inhibition of US28-mediated [ $^3\text{H}$ ]inositol phosphates production (Fig. 3). In contrast, coexpression of the  $\beta\gamma$ -scavenger  $\text{G}\alpha_t$  did not modify constitutive US28 signaling (Fig. 3).

These data indicate that, in contrast to the homologous CC chemokine receptor CCR1 (32), US28 interacts with endogenous  $\text{G}\alpha_{q/11}$  subunits in COS-7 cells and thereby constitutively activates phospholipase C. A large number of GPCRs can also couple to phospholipase C upon coexpression of  $\text{G}\alpha_{16}$ , an hematopoietic specific member of the  $\text{G}_q$  class of proteins (39). Expression of  $\text{G}\alpha_{16}$  enhanced, for example, the agonist-induced

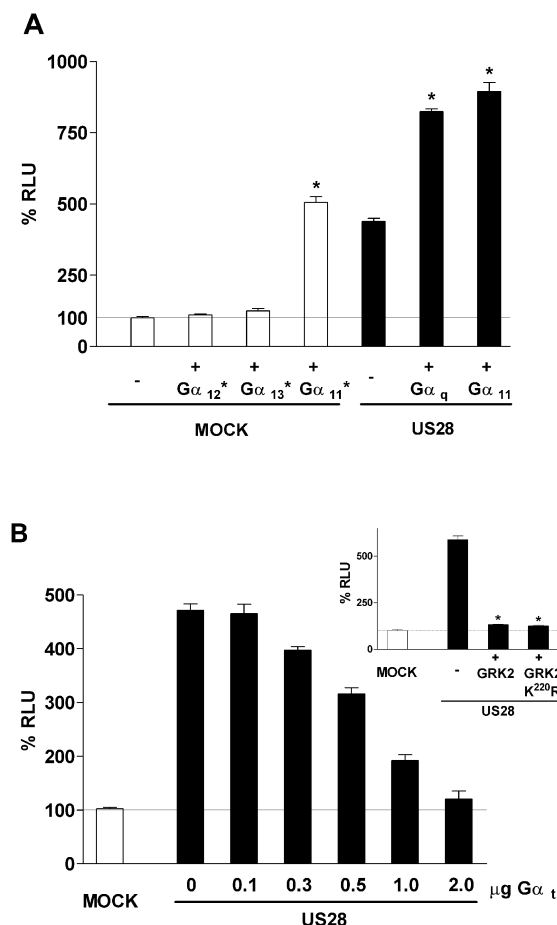


**FIG. 3. Effect of various  $\text{G}\alpha$  subunits on US28-mediated inositol phosphates accumulation.** COS-7 cells were transiently transfected with cDNA encoding US28 (2  $\mu\text{g}/10^6$  cells) in the presence of cDNAs encoding the indicated  $\text{G}\alpha$  subunits (2  $\mu\text{g}/10^6$  cells) and were assayed for InsP accumulation after 48 h. Expression of  $\text{G}\alpha$  subunits by themselves did not give a rise in [ $^3\text{H}$ ]inositol phosphate production (data not shown). *Inset*, the effect of coexpression of GRK2 and the mutant GRK2K<sup>220R</sup> or  $\text{G}\alpha$  transducin (4  $\mu\text{g}/10^6$  cells) on the US28-mediated InsP production. Data are expressed as percentage of US28-mediated response. Representative experiments performed in triplicate are shown, and each experiment was repeated at least three times. The asterisks indicate a statistically significant difference ( $p < 0.05$ ) versus receptor only.

inositol phosphate production mediated by the muscarinic m2 receptor 2.2-fold, as previously reported (40). However, US28 shows a remarkable level of selectivity for  $\text{G}\alpha_{11}$  and  $\text{G}\alpha_q$  over  $\text{G}\alpha_{16}$  for the coupling to phospholipase C in COS-7 cells.

Besides the US28-mediated modulation of phospholipase C activity, we also observed a constitutive activation of NF- $\kappa\text{B}$  activity upon expression of US28 in COS-7 cells (Fig. 1A). This effect was not observed for the homologous CCR1 receptor (data not shown). The constitutive stimulation of NF- $\kappa\text{B}$  activity was not modulated by RANTES or MCP-1 (up to 100 nM; data not shown), but fractalkine again behaved as an apparent partial inverse agonist. The US28-mediated increase in NF- $\kappa\text{B}$

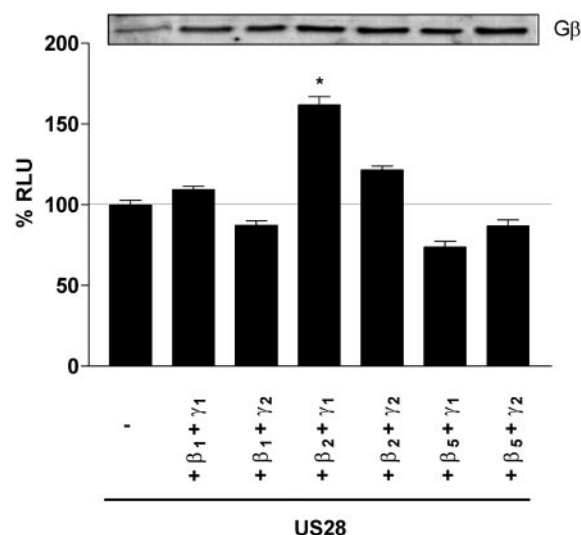




**FIG. 4. Effect of various  $G\alpha$  subunits on the NF- $\kappa$ B-driven luciferase expression.** A, COS-7 cells were transfected with either pcDNA3 (MOCK) or US28 ( $2 \mu\text{g}/10^6$  cells), together with cDNAs encoding different  $G\alpha$  subunits ( $2 \mu\text{g}/10^6$  cells); 48 h after transfection NF- $\kappa$ B-driven luciferase expression was measured. B, COS-7 cells were transiently transfected with US28 ( $2 \mu\text{g}/10^6$  cells) and increasing amounts of  $G\alpha_t$ . RLU, relative light unit. Inset, the effect of coexpression of GRK2 and the mutant GRK2<sup>K220R</sup> ( $2 \mu\text{g}/10^6$  cells) on the US28-mediated NF- $\kappa$ B activation. Data are expressed as percentage of control (mock cells). Representative results of three independent experiments performed in triplicate are shown. The asterisks indicate a significant difference ( $p < 0.05$ ) versus receptor only.

activity was reduced by fractalkine for  $42 \pm 4\%$  ( $n = 3$ ) with an  $\text{IC}_{50}$  value of  $5 \pm 0.2 \text{ nM}$ .

The observed constitutive activation of NF- $\kappa$ B by US28 is of potential pathophysiological relevance, as NF- $\kappa$ B is an ubiquitously expressed transcription factor that plays a critical role in the regulation of inducible genes in immune response and inflammatory events associated with, for example, atherosclerosis (41–43). Activation of NF- $\kappa$ B-mediated transcription has been reported in human aortic smooth muscle cells after CMV infection via PTX-sensitive G proteins. Yet, as observed for the production of [ $^3\text{H}$ ]inositol phosphates,  $G\alpha_i$  is not involved in the US28-induced increase in NF- $\kappa$ B activity, as PTX treatment did not affect the US28 response ( $104 \pm 5\%$ ;  $n = 3$ ). Previous studies have indicated that depending on the cell type and GPCR, NF- $\kappa$ B-mediated transcription can also be stimulated following activation of  $G_{12/13}$  or  $G_{q/11}$  proteins (44). Yet, in COS-7 cells, only the expression of the activated form of  $G\alpha_{11}$  ( $G\alpha_{11}$  Q209L, referred to as  $G\alpha_{11}^*$ ) subunits resulted in significant activation of NF- $\kappa$ B (Fig. 4A). In line with these findings, coexpression of the wild-type  $G\alpha_{11}$  or  $G\alpha_q$  (Fig. 4A) increased the US28-mediated constitutive NF- $\kappa$ B signaling, whereas the basal NF- $\kappa$ B activity in mock-transfected cells was not affected. These observations clearly imply the involvement



**FIG. 5. Effect of different  $\beta\gamma$  combinations on US28-mediated NF- $\kappa$ B activation.** COS-7 cells were transiently transfected with US28 ( $2 \mu\text{g}/10^6$  cells), NF- $\kappa$ B-Luc, and different combinations of  $G\beta_1$ ,  $\beta_2$ , and  $\beta_5$  with  $G\gamma_1$  and  $\gamma_2$  ( $2 \mu\text{g}/10^6$  cells of each construct); 48 h after transfection NF- $\kappa$ B-driven luciferase expression was measured. Data are expressed as percentage of US28-mediated response. A representative experiment performed in triplicate is shown, and each experiment was repeated at least three times. The asterisk indicates a significant difference ( $p < 0.05$ ) versus receptor only. RLU, relative light unit. Inset, expression of  $G\beta$  subunits measured by an antibody directed at a common motif in  $G\beta$ .

of the  $G\alpha_{q/11}$  proteins in the US28-mediated signaling to NF- $\kappa$ B. As found for the activation of phospholipase C, GRK2 and its kinase-deficient mutant inhibited the increase in NF- $\kappa$ B activity (Fig. 4B, inset). Yet, in contrast to the US28-induced phospholipase C activation, US28-mediated NF- $\kappa$ B activity was fully inhibited by coexpression of  $G\alpha_i$  (Fig. 4B). The involvement of  $\beta\gamma$  subunits in constitutive US28 signaling to NF- $\kappa$ B was further strengthened by coexpression experiments with various  $\beta\gamma$  subunits (Fig. 5, inset). Of the different combinations tested, only the  $G\beta_2$  and  $G\gamma_1$  further significantly increased the US28-mediated activation of NF- $\kappa$ B (Fig. 5). These data corroborate previous findings that GPCRs can show a clear specificity for specific  $\beta\gamma$  subunit combinations (45).

Whereas US28 activates phospholipase C via  $G\alpha_{q/11}$  subunits, our data suggest that besides  $\alpha_{q/11}$  subunits,  $\beta\gamma$  subunits are also involved in the NF- $\kappa$ B activation by US28. The apparent coinvolvement of  $\alpha_{q/11}$  and  $\beta\gamma$  subunits suggests that NF- $\kappa$ B activation is due to  $\beta\gamma$  subunits that are released upon US28 interaction with  $G_{q/11}$  proteins, although release of  $\beta\gamma$  subunits from other G proteins cannot be ruled out. As previously observed after stimulation of the bradykinin  $B_2$  receptor in Hela cells (44),  $G\beta\gamma$  subunits appear to be essential but not exclusive signaling moieties for the NF- $\kappa$ B signaling by US28. Expression of the various  $G\beta\gamma$  subunits by themselves did not increase NF- $\kappa$ B signaling (data not shown). Moreover, our observation that expression of activated  $G\alpha_{11}$  initiates NF- $\kappa$ B signaling indicates that  $G\alpha_{q/11}$  subunits also trigger a signaling pathway that converges to NF- $\kappa$ B. Protein kinase C activation is a likely candidate for this  $\alpha_{q/11}$ -mediated pathway (46). Activation of NF- $\kappa$ B via  $\beta\gamma$  subunits probably involves the activation of phosphatidylinositol 3-kinase and Akt (44), two recently identified signaling partners for GPCRs (47, 48). Additional experiments need to be performed to further delineate the mechanisms of US28-mediated NF- $\kappa$ B activation. The specific roles of the  $\alpha_{q/11}$  and  $\beta\gamma$  subunits, especially, will require further clarification.

In conclusion, in comparison to its closest human homologue

CCR1, it is interesting to note that the viral GPCR US28 signals without the need for an agonist and is using a larger diversity of G proteins and chemokines to affect cellular signaling pathways. For the first time, we show that US28 signals to phospholipase C via  $G_{\alpha_{q/11}}$  subunits and NF- $\kappa$ B via both  $G_{\alpha_{q/11}}$  and  $G_{\beta\gamma}$  in a constitutively active manner. It is tempting to speculate that these characteristics of a promiscuous GPCR allows US28 to affect a broad range of cells upon CMV infection. The constitutive activation of the ubiquitous transcription factor NF- $\kappa$ B by the early viral gene product US28 could be of major importance for viral action. US28 has been shown to cause smooth muscle cell migration upon HCMV infection without the addition of exogenous chemokines (31). The basal US28-mediated migration was antagonized for 80% by neutralizing antibodies against MCP-1, which was released in an autocrine fashion (31). Constitutive signaling by US28 could be responsible for the remaining migratory response of the HCMV-infected smooth muscle cells. Moreover, if US28 is expressed on viral particles, it would also be immediately present on the membrane of CMV-infected cells and, by means of its constitutive activity, could modulate the cellular response. We also show for the first time a functional response to the CX<sub>3</sub>C chemokine fractalkine, *i.e.* acting as an inverse agonist at US28. Fractalkine is a quite unique GPCR ligand as its chemokine-like domain is linked to a transmembrane segment (49). Accordingly, fractalkine is membrane-bound, and its interaction with US28 has been suggested to be involved in the viral transfer between cells (27). The action of fractalkine as an inverse agonist suggests that inhibition of constitutive US28 activity by fractalkine expressed on the membrane of a target cell might give the appropriate signal to an CMV-infected, US28-expressing cell to allow CMV entry into the target cell. Because no data are currently available on the expression of US28 on the viral particle or on the role of fractalkine in CMV infections, future investigations should substantiate these suggestions and indicate if US28 can be regarded as an interesting drug target in HCMV-related disorders.

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